THE PRODUCTION AND MEASUREMENT OF COLLOID CLOUDS IN ALCOHOLIC SOLUTIONS OF BLOOD SERUM LIPIDS[1]

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The inadequacy of our understanding of a pathological process is reason enough to stimulate newer attempts at its study. The group of clinical diseases ascribed to lipid dyscrasias stand high in the list of those most provocative of newer approaches. The use of biochemical methods in the study of these dyscrasias by fractional analyses of blood has been widely explored and has contributed much to our understanding of the lipid diseases. This report deals with the presentation of a biochemical technic for the study of blood lipids, based upon the fact that these lipids are not in solution in blood but are colloidally dispersed therein. This physical state brings up the question of the stability of these dispersions and the influence of a relative instability of them on the deposition of blood lipids in tissue.

Cholesterol is a particularly important compound in the lipid group, not alone from a clinico-pathological aspect, but also from a physico-chemical viewpoint. Using the procedure hereinafter outlined for dispersing as a colloid the lipids from an alcoholic extract of the blood, an alcoholic solution of as little as 0.1 mgm. of cholesterol per cc. cannot be colloidally dispersed; that is to say, flocculation[2] invariably results in this simple alcoholic solution which contains no blood serum lipids. On the other hand, the addition of ten times this minimum of cholesterol to the alcoholic blood lipid extract, while influencing the resultant dispersion cloud, does not effect flocculation. Thus far, studies made with the alcoholic extracts from normal blood reveal the capacity of such extracts to accept the addition of approximately three times the amount of free cholesterol natively present and react with a colloid cloud formation, using the technic here described. On the other hand, the addition to these extracts of ten times the amount of free cholesterol natively present invariably leads to flocculation. Our procedure in these blood lipid studies involves the production and measurement of colloid clouds in alcoholic extracts of blood serum with and without the addition to these extracts of alcoholic solutions of cholesterol.

PROCEDURE

Collection of Sample: Measurement is made on blood serum. The serum is removed with a pipette after the clot has formed and the tube containing the blood has been submitted to medium speed centrifugation. The serum may be kept in the ice box for as long

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[2] By flocculation is meant the occurrence of particles which, on agitation of the tube, show independent motion within the previously present stable colloid cloud.

as forty-eight hours before analysis, without influencing results. It is our procedure, however, to make analyses within eighteen hours after collection of the samples.

Care of glassware. Particles of all kinds, dirt, oil or water, may influence the nature of the colloid cloud formed in the reaction test tube. For this reason, all parts used in the cloud production, test tubes and glass stirring rods, must be kept scrupulously clean. Such glassware, therefore, is always cleaned with sulphuric acid-chromate solution, thoroughly rinsed in distilled water and allowed to dry overnight in preparation for use.

TECHNIC

To 0.5 cc. of blood serum, in an adequate sized tube, is added 10 cc. of 95% ethyl alcohol. The mixture is allowed to stand for half an hour, with frequent vigorous stirrings. The stirring rod has its end bent into a ring just large enough to fit into the test tube. This makes effective stirring possible by an up and down motion of the rod. The extract is then filtered through a Whatman grade 1 filter paper. Four samples of 0.8 cc. each of this filtrate are then placed in test tubes ($\frac{3}{4}''$ by $5\frac{7}{8}''$). To two of these is added 0.2 cc. of 95% ethyl alcohol and to two is added 0.2 cc. of a 0.05% alcoholic solution of cholesterol.

The colloidal emulsification of the lipid solutions is secured by adding a solution of 0.85% sodium chloride in distilled water dropwise from a burette to the alcoholic extract under continuous mechanical stirring of the extract. The total amount of chloride solution added is 5 cc. and it is added in the following fashion: The first 2 cc. at the rate of one drop every 3 to 4 seconds; the next 2 cc. at the rate of one drop every 2 seconds and the last 1 cc. at one drop per second. The emulsions are allowed to stand until all air bubbles have disappeared before readings are made. As the stirring rods must be perfectly clean and dry, a separate one is required for each sample. The tubes in which the emulsifications are produced are first placed in a water bath at 40°C., which temperature is maintained throughout by the use of a low bunsen flame. Furthermore, the extract is kept at this temperature for at least five minutes before the emulsification is started. The colloid clouds are read through a Duboscq form of colorimeter, using 1.5% $CoSo_4 \cdot 7H_2O$ as a standard solution, with a green filter (Wrattan #74) in the eye piece. For satisfactory readings the standard may be set most conveniently at 15 mm.

COMMENTS ON PROCEDURE

The use of twenty volumes of 95% ethyl alcohol as the total lipid extractive proved to give the same results as could be secured with the use of an ether-alcohol mixture, and for our purposes was obviously preferable, if not actually necessary (ether in the presence of an open flame). It was definitely demonstrated that temperature variations of the room interfered with the procurement of reproducible results. The use of a water bath at 40°C. overcame this difficulty. The dropwise method of adding the chloride solution and in the manner indicated is *absolutely essential.* It should be emphasized again that perfectly clean and dry glassware is imperative. Measurements must always be done in duplicate; uncontrolled influences in the production of colloid emulsions are an ever present threat. The color of cobalt sulphate appears to be a physiologic complement of the green in the Wrattan filter used. It serves, therefore, to produce variations of gray (opacity) interfering with light transmission as does the colloid cloud. Checks with an alcoholic solution of oleic acid as a source of a standard colloid emulsion revealed this artificial standard as completely adequate and acceptable. The clouds induced resist disruption by high speed centrifugation for fifteen minutes and give no change in reading after an eighteen hour interval of standing at room temperature.

Table I lists the analyses of forty eight samples of blood. The donors were ambulatory dermatologic patients, all adults and free of any known lipid dyscrasia. These samples were all chance samples and therefore the time of their taking bore no relationship to that of the last ingestion of food. This was intentionally done, to permit the influence of absorbed lipids in transit to make

TABLE I

Readings of emulsion clouds formed in lipid extracts with and without the addition of cholesterol

CASE NO.	0.8 CC. EXTRACT PLUS 0.2 CC. ALCOHOL		0.8 CC. EXTRACT PLUS 0.2 CC. ALCOHOLIC SOLUTION 0.05% CHOLESTEROL		AVERAGE READING DIFFERENCE (−) DUE TO ADDED CHOLESTEROL	CASE NO.	0.8 CC. EXTRACT PLUS 0.2 CC. ALCOHOL		0.8 CC. EXTRACT PLUS 0.2 CC. ALCOHOLIC SOLUTION 0.05% CHOLESTEROL		AVERAGE READING DIFFERENCE (−) DUE TO ADDED CHOLESTEROL
	Sample 1	Sample 2	Sample 1	Sample 2			Sample 1	Sample 2	Sample 1	Sample 2	
	16.3	17.1	13.3	12.9		21	33.3	31.7	23.1	24.5	8.7
1	16.5	17.4	13.3	12.6	3.8	22	29.7	29.1	23.7	23.8	5.6
	16.2	17.3	13.1	12.7		23	22.2	22.5	17.4	18.0	4.7
Av.	16.3	17.3	13.2	12.7		24	19.2	18.9	16.0	16.0	3.1
	18.4	18.2	13.3	14.3		25	23.5	23.5	18.8	20.3	3.9
2	18.2	18.1	13.3	14.4	4.4	26	15.5	16.0	13.7	13.6	2.1
	18.4	17.9	13.3	14.4		27	24.5	24.7	18.6	18.4	6.1
Av.	18.3	18.1	13.3	14.4		28	27.9	31.4	21.2	20.1	9.0
	14.3	14.2	11.7	11.5		29	21.0	21.2	18.1	18.3	2.9
3	14.3	14.1	11.8	11.3	2.7	30	15.0	15.9	13.7	13.8	1.7
	14.6	14.2	11.6	11.6		31	11.7	11.3	9.9	10.6	1.2
Av.	14.4	14.2	11.7	11.5		32	25.6	26.1	21.7	20.3	4.9
4	15.6	16.6	13.2	13.2	2.9	33	32.0	31.3	24.6	22.0	8.4
5	25.4	25.6	17.7	17.0	8.1	34	25.7	22.6	17.3	17.9	6.6
6	21.9	21.3	16.7	17.1	4.7	35	27.7	26.9	19.6	21.4	6.8
7	19.3	18.3	14.1	14.4	4.5	36	31.8	28.3	22.1	21.5	7.9
8	23.4	23.6	16.8	16.6	6.8	37	22.8	19.7	19.0	16.6	3.0
9	21.0	21.6	16.4	15.6	5.2	38	16.9	16.1	14.7	14.1	2.1
10	19.2	22.6	13.7	13.5	7.3	39	23.8	25.7	19.7	19.6	5.1
11	30.6	30.3	22.9	23.2	7.4	40	19.7	21.0	17.1	17.6	3.0
12	26.4	26.5	20.7	20.7	5.8	41	17.1	16.7	14.1	14.2	2.4
13	26.3	28.2	20.9	19.1	7.3	42	17.7	18.3	13.6	14.0	4.2
14	31.1	30.0	21.1	22.3	8.9	43	15.0	14.0	12.2	12.3	2.2
15	25.1	24.6	21.1	21.7	5.5	44	21.4	21.9	17.9	18.8	3.3
16	17.9	17.8	13.1	13.3	4.7	45	24.8	24.0	19.2	19.9	4.8
17	26.3	27.3	19.9	19.5	7.1	46	21.6	21.4	16.7	16.9	4.7
18	31.7	29.8	22.7	23.0	7.9	47	13.4	14.3	11.7	12.3	1.9
19	19.2	18.9	17.7	17.8	1.2	48	28.3	27.9	22.2	21.7	6.1
20	27.9	28.0	24.2	23.7	4.0						

their impression on the range of measurements. We realize that in the ultimate evaluation of this procedure for the clinic, the samples to be studied had best eliminate this influence by accepting blood taken only in the post-absorptive state.

The data reveal: 1) The measurements of duplicates and 2) the influence of

added cholesterol on the colloid cloud formed. The majority of duplicate samples give readings that check excellently. The failure of the few to check within the limits expected for a colorimetric technic, may be explained by the presence of uncontrollable minor variables influencing the colloid cloud formation.

TABLE II

Measurements on varying amounts of the same extract

SAMPLE NO.	AVERAGE MEASUREMENT OF DUPLICATES			CALCULATED READINGS FOR 1 CC. EXTRACT BASED ON MEASUREMENT OF INDICATED FRACTION		
	Amount of extract					
	0.4 cc.	0.6 cc.	0.8 cc.	0.4 cc.	0.6 cc.	0.8 cc.
1	22.0	15.6	11.7	8.9	9.3	9.3
2	31.8	21.1	15.5	12.7	12.7	12.4
3	22.6	16.6	11.8	9.1	9.9	9.4
4	33.8	21.6	17.7	13.5	13.0	14.2
5	30.8	20.6	15.9	12.3	12.5	12.7
6	18.8	13.1	10.2	7.5	7.9	8.1
7	20.0	14.5	12.4	8.0	8.7	9.9
8	30.2	18.9	14.7	12.1	11.3	11.9
9	26.9	17.0	14.1	10.8	10.2	11.2
10	22.6	15.2	11.9	9.0	9.1	9.5
11	29.1	19.7	14.2	11.6	11.8	11.4
12	27.6	18.0	13.4	11.0	10.8	10.7
13	28.4	19.2	14.1	11.4	11.5	11.3
14	24.8	17.2	13.1	9.9	10.3	10.4
15	20.8	14.4	10.6	8.4	8.6	8.5
16	20.2	13.2	10.0	8.1	7.9	8.0
17	24.1	16.6	11.7	9.7	9.9	9.4
18	25.0	16.7	12.1	10.0	10.0	9.7
19	21.7	14.7	10.7	8.7	8.8	8.6
20	26.4	17.5	13.1	10.5	10.5	10.5
		0.8 cc.	1.0 cc.		0.8 cc.	1.0 cc.
21		21.5	16.4		17.2	16.4
22		24.8	16.8		16.6	16.8
23		13.5	10.9		10.8	10.9
24		15.3	12.3		12.2	12.3
25		21.7	16.1		17.3	16.1
26		21.1	16.8		16.9	16.8
27		16.6	12.6		12.2	12.6
28		15.1	12.5		12.1	12.5

Table II supplies estimations on varying quantities of the same extract for revealing the quantitative character of the reaction. The data completely justify the conclusion that within the quantitative range adaptable to the technic, the colloid clouds formed are proportional in opacity induced to the amount of extract in which they are produced.

The data in Table III include, in addition to the measurement of the colloid clouds provoked, the measures of the several lipid fractions for each of the samples analyzed. Further, data are given of the measurement or effects, as referable to the subsequent emulsification induced, of the addition to each blood extract of three different quantities of an alcoholic solution of cholesterol.

All twenty-five samples were from ambulatory dermatologic patients, all of

TABLE III

SAMPLE NUMBER	0.8 cc. A, 0.2 cc. C, AVERAGE READING	0.8 cc. A, 0.2 cc. B, AVERAGE READING	0.7 cc. A, 0.3 cc. B, AVERAGE READING	0.65 cc. A, 0.35 cc. B, AVERAGE READING	0.5 cc. A, 0.5 cc. B, AVERAGE READING	TOTAL LIPID	FATTY ACID	LIPOID PHOSPHORUS	TOTAL CHOLESTEROL	ESTERIFIED CHOLESTEROL	ESTERIFIED CHOLESTEROL
						mgm. %	*mgm. %*	*mgm. %*	*mgm. %*	*mgm. %*	*per cent*
1	21.9	17.1			Flocc.	518	238	3.3	270	185	68
2	21.4	17.8			Flocc.	319	105.5	4.8	205	140	68
3	16.5	14.0			Flocc.	1082	833	7.9	240	165	69
4	29.0	21.8			Flocc.	1090	864	2.6	150	95	63
5*	8.5	8.1			Flocc.	2032	1640	14.9	380	210	55
6	14.6	12.8		14.7	Flocc.	477	194	3.2	270	170	63
7	26.2	21.0		Flocc.	Flocc.	450	259	4.3	185	125	68
8	32.3	26.3				468	287	4.1	175	85	49
9	31.2	26.9		Flocc.	Flocc.	486	305	2.9	175	125	71
10	20.4	17.4		Flocc.	Flocc.	568	352	4.6	210	110	52
11	23.0	18.9		Flocc.	Flocc.	558	389	4.0	155	90	58
12	21.7	16.9		Flocc.	Flocc.	541	314	4.0	220	120	54
13	21.1	17.7	19.9	Flocc.		486	268	4.0	210	135	64
14	16.6	13.9	15.5	Flocc.		648	305	6.2	335	225	67
15	22.6	19.6	Flocc.	Flocc.		486	194	4.0	280	140	50
16	25.1	20.8	Flocc.	Flocc.		747	537	5.7	210	110	52
17	24.7	18.8	Flocc.	Flocc.		414	185	6.5	220	120	55
18	15.5	13.1	15.5	Flocc.		486	213	5.6	250	145	58
19	27.1	19.0	Flocc.	Flocc.		540	305	5.6	225	145	64
20	28.8	21.9	Flocc.	Flocc.		531.5	324	4.9	200	80	40
21	17.8	15.5	16.8	Flocc.		504.5	296.2	8.0	205	125	61
22	18.6	15.6	21.2	Flocc.		423.4	213	7.5	205	135	66
23	30.2	22.5	Flocc.	Flocc.		396.3	203.7	5.7	180	105	58
24	20.1	19.5	Flocc.	Flocc.		675.6	472	6.9	200	135	68
25	25.2	19.3	Flocc.			486	277	7.0	205	80	39

A = alcoholic extract of blood; B = .05% cholesterol in alcohol; C = 95% ethyl alcohol.
* Case of xanthomatosis cutis.

whom except one (Case 5: generalized xanthomatosis cutis) were free of any known lipid dyscrasia. All samples were chance samples and the time of taking them unrelated to the moment of the last ingestion of food. This was again intentionally done to permit the widest influence of absorbed lipids in transit on all measurements. All analyses and procedures for each sample were done on a single extract from that sample, which was made by the addition of twenty volumes of 95% ethyl alcohol to blood serum, as given above. Reference to the

technical procedures used in estimating the several lipid fractions are given below:[3]

Table III lists four different ratios in the mixture of the blood extract and cholesterol solution, 0.05% in alcohol: 1) 0.8 cc. extract plus 0.2 cc. cholesterol solution; 2) 0.7 cc. extract plus 0.3 cc. cholesterol solution; 3) 0.65 cc. extract plus 0.35 cc. cholesterol solution; and 4) equal volumes (0.5 cc.) of each. Two or three of these ratios were used in the analysis of each sample. In all samples where equal volumes of blood extract and cholesterol solution were used, a pure colloid cloud could never be produced. Flocculation occurred regularly. On the other hand, in the mixture of 0.8 cc. blood extract and 0.2 cc. cholesterol solution, a pure colloid cloud occurred regularly. Measurements revealed that a cloud of increased opacity always resulted from this addition of cholsterol solution to blood extract. The ratios 0.7 cc. blood extract and 0.3 cc. cholesterol solution, and 0.65 cc. blood extract and 0.35 cc. cholesterol solution, where used usually resulted in the formation of a flocculate. Five samples (numbers 13, 14, 18, 21 and 22) of the former ratio, and one (number 6) of the latter ratio, apparently supplied a pure colloid cloud. This we inferred from the failure to see in these samples any independent motion of a flocculence amidst the stable cloud. However, it will be noted from the measurement of these samples that they showed less opacity than the corresponding samples containing the mixture 0.8 cc. extract and 0.2 cc. cholesterol solution; and in one case less of an opacity than that induced in the extract alone. Since the progressive increase of added cholesterol to lipid extracts increases the opacity of the colloid cloud induced until the point of flocculent precipitation, we were in these few samples presented with a reversal of this effect—a sort of clearing phenomenon. Studies made to substantiate the presence of such a clearing phenomenon revealed that this apparent clearing was due to a discharge from the mixture of imperceptible flocculent precipitates on production of the colloid cloud. In other words, evidence of a reversal of the tendency for the presence of relatively increased amounts of cholesterol to produce greater opacity in a colloid cloud was proof of the earliest production therein of flocculation.

A study of the colloid densities induced in the blood lipid extracts alone showed variations in measurement ranging over 100% (Case 5. of xanthomatosis cutis ignored). Furthermore, these measurements showed no apparent correlation with the total lipids in the sample. Thus sample #4, with a high total lipid of 1090 mgm. per cent had a colloid reading of 29, and sample #23, with a total lipid of 396.3 mgm. per cent had a colloid reading of about the same, 30.2; and sample #6, with a high density cloud reading, 14.6, had a modest total lipid of 477 mgm. per cent. It can be accepted definitely that the density of the colloid

[3] Reference to technic used in lipid fraction estimations:

Total lipids and fatty acids: Bloor, W. R., Jour. Biol. Chem., **77**: 53, 1938.

Cholesterols and cholesterol esters: Bloor, W. R., and Knudson, A., Jour. Biol. Chem., **27**: 107, 1919; Ibid., **29**: 7, 1917.

Phosphatid: Whitehorn, J. C., Jour. Biol. Chem., **62**: 133, 1924.

clouds formed are functions of the composite character of the lipids in the sample and not simply of the arithmetical total of these lipids.

Similarly, a comparison of the degrees of influence on the density measurement of the colloid cloud by the addition to each 0.8 cc. of the lipid extract of 0.2 cc. cholesterol solution, leads with equal assurance to the conclusion that the assembled and relative characteristics of the lipids and not their sum total in the sample is the controlling factor. And so cases #3 and #4, with total lipids approximately the same, not only give significantly different opacities in the colloids from the lipid extracts alone but show the decidedly different influence on them by the addition of cholesterol, the former showing an increase in density of 2.5 and the latter of 7.2.

We may comment briefly on the single pathological case in the group—#5, *xanthomatosis cutis*. This patient had literally hundreds of pea-size and larger xanthomas scattered over tunk and extremities. The blood serum was extremely lactescent. The lipid fractions were abnormally high throughout, as was anticipated from the appearance of the serum. The colloid cloud was extraordinarily dense (8.5) as might be expected; but significant was the failure of added cholesterol to increase that density (8.1).

These data support the belief that the quantitative character of the colloid clouds produced is a function of the lipid extract taken as a whole. While it may be true that, as with the addition of increasing amounts of solutions of cholesterol, factors forming increased colloid opacities may effect flocculation, there is no reason to assume that, based on a measurement of the opacity of the formed colloid cloud, we can pass judgment on the stability of the lipid suspension in the original blood sample. Nor can we assume that this judgment can necessarily be accomplished by noting the points of effected flocculation with added cholesterol or other substances. The significance of these opacity measurements and flocculent formation must await studies of this procedure on bloods in a variety of diseased states.

These data justify the presumption that our technic of blood lipid study reveal a biologic characteristic of the blood not previously available to quantitative studies. This new characteristic should be probed for its meaning and possible clinical helpfulness. Much has been written about the blood lipids as colloids and the degrees of their stability in the blood stream, as important among the etiologic factors in the pathogenesis of the several lipid dyscrasias. Many attempts have been made, using lipid fractions of the blood, to find a correlation between fractions as revealing degrees of stability of the lipids in the circulation. Already data[4] are available emphasizing the need for judging analysed lipid fractions as related one to the other rather than as independent absolute quantities, in the task of keeping the total lipids stably suspended in the blood. The use of our procedure may throw much light on all these approaches. The limited number of analyses here recorded, the use of chance samples as against the necessary practice of taking them in the post-absorptive state, and the use of

[4] Schaaf, Fritz, Jour. Inves. Dermat., **1**: no. 1, p. 11, Feb., 1938.

alcohol alone in contrast to the usual alcohol-ether mixture for extracting the lipids, make futile an attempt to correlate the so-called "stability indices" derived from the lipid fractions and the colloid densities in the samples herein reported. The value of such a study, however, seems definitely indicated. Nor can one ignore the possible value of using some lipid other than cholesterol for influencing the opacities induced or for the production of flocculation.

This report is given at this time to make available to other workers this procedure for blood lipid studies. We plan to make further studies, using this method, in the attempt to throw new light on the problem of lipid metabolism.